Genotypic and Phenotypic Characterization of Aerosolized Bacteria Collected From African Dust Events

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Twenty-one bacteria were isolated and characterized from air samples collected in Africa and the Caribbean by the United States Geological Survey (USGS). Isolates were selected based on preliminary characterization as possible pathogens. Identification of the bacterial isolates was achieved using 16S rRNA gene sequence analysis, fatty acid methyl esters (FAMEs) profiling, the BIOLOG Microlog® System (carbon substrate assay), and repetitive extragenic palindromic (REP)-PCR analysis. The majority of isolates (18/21) were identified as species of the genus *Bacillus*. Three isolates were classified within the *Bacillus cereus senso lato* group, which includes *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus cereus* strains. One isolate was identified as a *Staphylococcus* sp., most closely related to species (i.e., *Staphylococcus kloosii*, *Staphylococcus warneri*) that are commonly associated with human or animal skin, but can also act as opportunistic pathogen. Another isolate was tentatively identified as *Tsukamurella inchonensis*, a known respiratory pathogen, and was resistant to the ten antibiotics tested including vancomycin.

Introduction

Aerosolized microorganisms are a rising concern affecting public health. Several pathogenic microorganisms are transmitted through airborne transport. Pathogenic species of the genera *Bacillus*, *Yersinia, Francisella, Legionella* and *Salmonella* are among a few that impact public health and ecological issues. Microorganisms can be aerosolized by wind or water as spores, cells or rafted on dust particles (Kuske 2006)*.* Long-distance air dispersal of these microorganisms is important for survival. *Puccinia melanocephala*, the causative agent of sugarcane rust, produces uredospores that have demonstrated aerial dispersal on a continental scale (Brown and Hovmoller 2002). In a recent study the causative agent of Legionnaires disease, *Legionella pneumophila*, was found to have infected persons in a 6 km radius from the contaminated industrial cooling tower (Nguyen et al. 2006). Species of *Bacillus* and other genera have been collected in the atmosphere at an altitude of 20 km (Griffin 2004) and in the stratosphere at an altitude of 41 km (Wainwright et al. 2003). Research has shown that microorganisms aerosolized into the atmosphere are capable of surviving long-range transport on a global scale with public health implications (Griffin, 2008). Disease outbreaks, including meningococcal meningitis, have been associated with dust storm activity (Garrison et al. 2003). Dust clouds originating from West Africa transport large quantities of airborne soil across the Atlantic by the tropical trade winds each year (Griffin et al. 2003). African dust storm activity across the Atlantic has been increasing for the past thirty years and it has been hypothesized that pathogenic microorganisms are being transported as far as the Caribbean and Americas (Griffin et al. 2001). *Aspergillus sydowii*, a fungal pathogen of gorgonian corals in the Caribbean, has been extensively studied in relation to African dust storms (Weir-Brush 2004). These studies have shown the importance of monitoring long-range transport of microorganisms in the atmosphere during dust storm activity.

Accurate and rapid characterization of aerosolized microorganisms is essential in the biomonitoring of the Earth's atmosphere. Several genotypic and phenotypic methods have been used to analyze cultured bacteria collected from the environment and identify possible pathogens. Analysis of the 16S rRNA gene sequence is one of the most commonly used methods of identification of bacteria. However, studies have shown that several species have high similarities in the sequence and can cause false identification (La Duc et al. 2004; Bavykin et al. 2004). REP-PCR has been recognized as an effective method for subspecies classification and strain delineation of bacteria. The method involves primers that target noncoding repetitive sequences throughout the genome and generate reproducible characteristic patterns when separated on an agarose gel (Healy et al. 2005; Szczuka and Kaznowski 2004). The

Sherlock Microbial Identification System analyses and identifies isolates using sample preparation procedures and gas chromatography to produce FAME profiles that are compared to profiles of known strains. This method has been successfully applied to the identification of bacteria and current methods allow for fast analysis (Whittaker et al. 2003). The BIOLOG® Microlog® Bacterial Identification System is a 95 carbon substrate utilization test that consists of specialized 96 well plates that are read by a fiber optic instrument. The BIOLOG® system has demonstrated the ability to accurately identify environmental bacteria (Roach et al. 2006). The purpose of this study was to identify isolated bacteria from air filters collected in Africa and the Caribbean during various dust events. Characterization of these and other isolates will allow us to better understand the significance of African dust transport of microorganisms. Viability was a significant factor to evaluating the bacteria in these aerosol samples.

Materials and Methods

Environmental sampling and preliminary identification of bacterial isolates

Three environmental air samples and a control were collected at several sites in Mali West Africa, Trinidad and St. Croix during dust and non-dust events. Air was sampled for microorganisms with pre-sterilized 47mm diameter, 0.2 µm pore-sized filter membranes using a vacuum pump with an airflow rate of $18 - 33$ liters per minute for approximately 12 minutes (Griffin et al. 2001). Filters were shipped to the University of South Carolina Aiken (Mali samples were sent in diplomatic pouches), where they were divided then placed on glycerol artificial seawater (GASW), yeast extract glucose (YEG) and nutrient agar (NA) plates. Bacteria were isolated and initially characterized using Gram stain, malachite green spore staining, microscopy, and the BIOLOG Microlog® Bacterial Identification System (BIOLOG® Inc., Hayward, CA). Twenty-one bacteria from eight separate filters were selected, based on this preliminary BIOLOG analysis, as potential human and plant pathogens (Table 1). Isolates from Mali designated MAL-1 through MAL-19 were collected in Mali during dust events. Isolate STX-20 was collected in St. Croix during dust-related haze conditions and TRIN-21 was collected in Trinidad during a non-dust event.

REP-PCR analysis

A small plastic pipet tip was used to suspend a small amount (barely visible) of an individual bacterial colony in 10 µl of distilled water. A 25 µl PCR mixture contained 1 µl of the suspended bacteria, 250 µM deoxynucleoside triphosphates, 2 U of HotStar*Taq* DNA polymerase (QIAGEN Inc., Valencia, CA), 1mM MgCl₂ 0.1% dimethyl sulfide, and 50 pM concentrations of the opposing primers REP1R-1 (5' III ICG ICG ICA TCI GGC 3') and REP2-1 (5' ICG ICT TAT CIG GCC TAC 3') (de Bruijn 1992). The PCR program for amplification was as follows: 1 cycle at $95^{\circ}C$ for 10 min; 35 cycles at $90^{\circ}C$ for 30 s, $40^{\circ}C$ for 1 min and $65^{\circ}C$ for 8 min; 1 cycle at $65^{\circ}C$ for 16 min for the final extension. Products were visualized on a 1% agarose gel stained with ethidium bromide.

PCR amplification and sequence analysis of the 16S rRNA gene

PCR amplification of the 16S rRNA gene was performed with a 20 µl mixture that contained 1 µl of a bacterial suspension (see REP-PCR section above), 250 µM deoxynucleoside triphosphates, 0.75 U of HotStar*Taq* DNA polymerase, 10 µg BSA, and 100 pM concentrations of the opposing primers 16S-27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 16S-1492R (5' TAC GGT TAC CTT GTT ACG ACT T 3') (Bodour et al. 2003). Cycles for amplification were as follows: 1 cycle at $95^{\circ}C$ for 10 min; 30 cycles at $94^{\circ}C$ for 1 min, $50^{\circ}C$ for 30 s, $72^{\circ}C$ for 1 min; 1 cycle at $72^{\circ}C$ for 5 min. The product was visualized on a 1% agarose gel then extracted and purified using the QIAquick Gel extraction kit (QIAGEN Inc., Valencia, CA). Purified PCR products were directly sequenced on a CEQ 8000 automated sequencer (Beckman; Fullerton, CA USA) using Sanger chemistry and primers 16S-27F, 16S-907R (5' GCC CCC GTC AAT TCM TTT RAG TTT 3') (Weidner et al. 1996) or 16S-1492R. DNA sequences were trimmed for quality and contigs were constructed using LaserGene SeqManII ver. 5.07 software (DNAStar, Inc.; Madison, WI USA). Sequences of the isolates were compared to known sequences using BLAST and the Classifier and Seqmatch functions of the Ribosomal Database Project (Wang et al. 2007).

FAME (GLC) analysis

FAMEs were analyzed using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE) and compared to the TSBA40 library (version 4.1) of previously determined profiles. Manufacturer's instructions were followed for growth, harvesting, saponification, methylation, and extraction of the samples. FAMEs were analyzed on a Hewlett-Packard 6850 gas chromatograph with an auto-sampler.

BIOLOG identification

The BIOLOG® Microlog® Bacterial Identification system (BIOLOG® Inc., Hayward, CA) was used to analyze the isolates. Isolates were prepared and inoculated following the BIOLOG® Microlog® user guide. Isolates were grown on BIOLOG® BUG® agar and characterized by Gram staining. Inoculates were prepared by collecting cells with a sterile swab and suspending in 20 ml tubes of BIOLOG® GP/GN inoculation medium to a specific level of transmittance (20% transmittance for non-spore forming and 28% for spore forming) using turbidity standards. Thioglycolate was added to inocula (5mM final concentration) to decrease false positives. BIOLOG® plate growth results were analyzed 12 hours and 24 hours after inoculation with the BIOLOG® GEN II Data Collection Software®.

Table 1. Dates, sites, and conditions of filters from which suspected pathogenic isolates were recovered

Isolate	Filter	Date Collected	Site	Event
MAL-1,2	Mal-B # 3	3/2/2004	Mali (Emetteur Kati)	Dust
MAL-3,4,5,6	Mal-D # 3	2/17/2004	Mali (Emetteur Kati)	Dust
MAL-7,8,9,10,11,12,13	Mal-G # 1	4/6/2004	Mali (Emetteur Kati)	Dust
MAL-14,15	Mal-G # 2	4/6/2004	Mali (Emetteur Kati)	Dust
MAL-17	Mal-G # 3	4/6/2004	Mali (Emetteur Kati)	Dust
MAL-18,19,22	Mal-L # 2	3/21/2004	Mali (Abebara)	Dust
STX-20	Stx-H # 3	6/10/2004	St. Croix (Point Udall)	Haze
TRIN-21	Tri-JJ # 3	1/15/2005	Trinadad (Galera Pt Lighthouse)	No Dust

*Antimicrobial susceptibility and isolate characterizatio*n

E-tests (AB BIODISK®, Solna, Sweden) were performed following the manufacturer's instructions. Inoculates were prepared by collecting cells with a sterile swab and suspending cells in saline to a dilution matching a 0.5 McFarland turbidity standard. Mueller-Hinton agar plates (150-mm) were inoculated with the cell suspensions using a sterile swab, and five antibiotic strips with predefined gradients were then applied. A total of ten antibiotics were tested for each isolate. Plates were incubated at room temperature, and growth inhibition was determined over a three-day period. Isolates were further characterized using standard biochemical tests for oxidase and catalase activity, growth on 50% tryptic soy agar at 37°C for 48 hours, and hemolytic activity on sheep blood agar plates.

Results

Analysis of partial 16S rRNA gene sequences revealed that most (18/21) of the isolates belonged to the genus *Bacillus* (Table 2). All isolates tested were Gram-positive rods except for the MAL-6, which was a Gram-positive cocci (Table 3). Three isolates, MAL-8,13,21 were closely aligned with the *Bacillus cereus senso lato* group, which includes *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cereus sensu stricto*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides*. Members of this group share nearly identical 16S rRNA gene sequences. Indeed, the 16S rRNA gene sequence from MAL-8 was 100% identical to many members of this group. In contrast, the 16S rRNA gene sequence from MAL-13 and MAL-21 were 99% similar to known members of the *Bacillus cereus senso lato* group. From our data, it is not clear if these differences are due to sequencing errors or reflect true diversity within the group. The REP-PCR did not yield bands for these three isolate (Figure 1), thus further genetic delineation was not provided. It is likely that cells/spores of these strains did not lyse during the colony REP-PCR procedure, as REP-PCR patterns have been generated from total DNA obtained from various *Bacillus cereus sensu stricto* strains (Bartoszewicz et al. 2008). FAME and BIOLOG results support the placement of
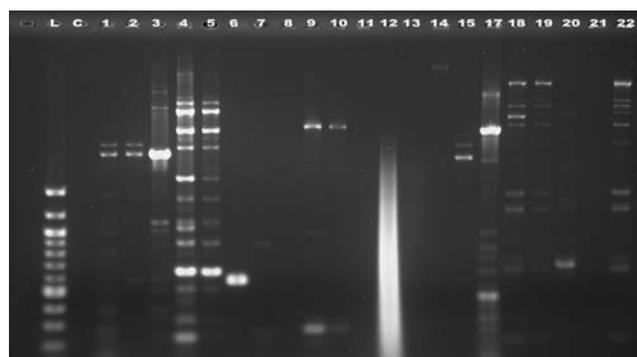

Figure 1. REP-PCR analysis of the isolates (Lane L, 1 Kb ladder; Lane C, negative control)

each of these 3 strains into the *Bacillus cereus sensu stricto* group*;* however, FAME classified TRIN-21 as *B. mycoides* and MAL-8, 13 as *B. cereus* (Table 2). Phenotypic evaluation further differentiated MAL-8, 13, 21 (Table 3). MAL-8 and MAL-13, were resistant to six of the antibiotics tested, whereas MAL-21 only exhibited intermediate resistance toward a single antibiotic, chloramphenicol. Furthermore, MAL-8 and MAL-13 caused hemolysis on sheep blood agar plates, but TRIN-21 did not. Nakamura and Jackson (1995) have previously demonstrated that the *B. mycoides* group could be distinguished from *B. cereus* by differences in fatty acid profiles.

One isolate, MAL-6, was identified as a *Staphylococcus* sp. and was most closely related to *Staphylococcus warneri* as determined by 16S rRNA gene sequencing (99% similarity) and *Staphylococcus kloosii* using FAME or BIOLOG® (Table 2). Both of these species are commonly associated with human or animal skin, but can also act as opportunistic pathogen (Kloos & Schleifer 1975; Schleifer et al. 1984). MAL-6 was resistant to tetracycline, showed intermediate resistance for chloramphenicol, and did not induce hemolysis on sheep blood agar plates (Table 3). Another isolate, STX-20, was tentatively identified (79% certainty) using BIOLOG® as *Tsukamurella inchonensis*, a known respiratory pathogen (Gomez, 2003). This isolate was notable in that is was resistant to the ten antibiotics tested including vancomycin (Table 3). STX-20 was also distinctive because it did not exhibit growth at 37°C, whereas all other strains did. This was of interest since STX -20 was the only isolate here from St.

Table 2. Characterization of the isolates by 16S rRNA, FAME, and BIOLOG®

Isolate	16s rRNA gene[1]	FAME	BIOLOG
MAL-1,2	*Bacillus niabensis* (99%)	*Paenibacillus apiarius*	*Paenibacillus polymyxa*
MAL-3	*Bacillus foraminis* (99%)	**Could not identify***	**Could not identify**[*]
MAL-4,5	*Bacillus thioparans* (99%)	**Could not identify**	**Could not identify**
MAL-6	*Staphylococcus warneri* (99%)	*Staphylococcus kloosii*	*Staphylococcus kloosii*
MAL-7	*Bacillus subtilis* (99%)	*Bacillus subtilis*	*B. subtilis*
MAL-8,13	*Bacillus cereus/thuringiensis/ anthracis* (99-100%)	*Bacillus cereus*	*B.cereus/thuringiensis*
MAL-9,10	*Bacillus fumarioli* (99%)	*Bacillus megaterium*	**Could not identify**
MAL-11	*Bacillus massiliensis* (99%)	**Could not identify**	**Could not identify**
MAL-12	*Bacillus licheniformis* (100%)	*Bacillus licheniformis*	*Bacillus licheniformis*
MAL-14	*Microbacterium sp.* (98%)	*Cellulomonas flavigena*	*Microbacterium* sp.
MAL-15	*Bacillus licheniformis* (99%)	*Bacillus licheniformis*	*Bacillus licheniformis*
MAL-17	*Bacillus firmus* (99%)	*Bacillus GC group 22*	*Bacillus firmus*
MAL-18,19,22	*Bacillus mojavensis* (100%)	*Bacillus subtilis*	*Bacillus subtilis*
STX-20	**Could not identify**	**Could not identify**	*Tsukamurella inchonensis*
TRIN-21	*Bacillus cereus/thuringiensis/ anthracis* (99%)	*Bacillus mycoides*	*B.cereus/thuringiensis*

Croix. Unfortunately, STX-20 did not produce PCR product for sequencing (using cell suspension material or genomic DNA as template) and the FAME results were inconclusive, thus its identity could not be confirmed.

Overall, FAME, BIOLOG®, and 16S rRNA gene sequence analysis yielded complimentary results. The most prominent exception was the identification of MAL-14 as a *Cellulomonas* sp. (*Cellulomonadaceae* family of the *Actinobacteria*) using FAME, whereas the same isolate was classified as a *Microbacterium* sp. (Microbacteriaceae family of the *Actinobacteria*) by 16S rRNA gene analysis and BIOLOG®. REP-PCR patterns of isolates determined to be closely related via the other methods (e.g. MAL-18, 19, 22; MAL-1, 2; MAL-4), were highly similar (Fig. 1). However, slight variations between the patterns of these closely related isolates could be detected, indicating genetic variation that could result in phenotypic differences, though none were detected in our limited analysis (Table 3).

Discussion

Most isolates were identified as *Bacillus* species, which are aerobic spore-forming gram positive rods that are associated with terrestrial habitats (Wu et al. 2006). While methods have been developed to deposit *Bacilli* spores via aerosolization onto surface materials for detection studies (Lee et al. 2013), these were collected onto 0.2 μM filters. The spores produced by the *Bacillus* species can be easily carried by winds and are relatively resistant to UV radiation (Wainwright et al. 2003) allowing for possible long range transport. Three potential pathogens were identified from the isolates studied. *B. cereus* is a ubiquitous species that causes food-borne illnesses and

has led to rare cases of fatalities (Dierick et al. 2005). *B. cereus* has also been found to cause wound infection, pneumonia, meninigitis and gastrointestinal infections. *B. cereus* is closely related to *B. anthracis*, the causative agent of anthrax, and *B. thuringiensis,* an insect pathogen (Sacchi et al. 2002). 16S rRNA analysis is not able to completely discriminate strains within the three species (La Duc). A *B. cereus* strain has also been identified to possess plasmids similar to the *B. anthracis* toxin encoding pXO1 plasmid. This strain causes an illness resembling inhalation anthrax (Hoffmaster et al. 2004). *B. anthracis* is typically not hemolytic as found here (Table 2). However, an atypical *B. anthracis* strain isolated from dead African chimpanzees and a gorilla in Côte d'Ivoire and Cameroon were found to be beta hemolytic (Klee et al. 2006).

B. licheniformis has been found to cause food poisoning along with other types of infections (De Clerck and De Vos, 2004). However this species is also important industrially in the production of enzymes and solvents (Wu et al. 2006). Another possible pathogen, *T. inchonensis,* has been found to cause pulmonary infections in immunocompromised patients (Gomez 2003). Two of the potential pathogens, *B. cereus* and *T. inchonensis,* were resistant to antibiotics. The other isolate that demonstrated microbial resistance was the *Staphylococcus* species.

Initial identification of bacterial isolates is commonly performed by analysis of 16S rRNA gene sequences. Only a trace amount of sample is needed and there is a large database to reference. However, the 16S rRNA gene of closely related species can be identical (i.e. the *Bacillus cereus sensu lato* group), and a phylogenetic representation using this method is based on just a single gene, which does not capture the phenotypic plasticity and genomic diversity within many species. Thus, further characterization is required (Bavykin et

Table 3 Antibiotic resistance and phenotypic characterization of the isolates.

Isolate (MAL, STX, or TRIN)	1, 2	3	4,5	6	7	8, 13	9, 10	11	12	14	15	17	18,19, 22	20	21
Benzylpenicillin	S	S	S	S	S	**R**	S	S	S	S	S	S	S	**R**	S
Ampicillin	S	S	S	S	S	**R**	S	S	S	S	S	S	S	**R**	S
Oxacillin	S	S	S	S	S	**R**	S	S	S	S	S	S	S	**R**	S
Cefotaxime	S	S	S	S	S	**R**	S	S	S	S	S	S	S	**R**	S
Vancomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S
Tetracycline	S	S	S	**R**	S	**R**	S	S	S	S	S	S	S	**R**	S
Chloramphenicol	S	S	S	I	I	I	S	I	S	S	I	S	I	**R**	I
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S
Ofloxacin	I	S	S	S	S	**R**	S	I	S	I	S	S	S	**R**	S
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Oxidase	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Hemolysis	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Gram Stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Morphology	rod	rod	rod	cocci	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod

al. 2004). FAME analysis and the BIOLOG Microlog® System are such tools that can aid in species identification when 16S rRNA gene analysis does provide a match to a closely related, cultured species. However these methods have a limited database and require a large amount of biomass. Furthermore, some genera are similar in biochemistry (an example in this study was the *Microbacterium* species that was identified as a *Cellulomonas* species) and can cause false identification in phenotypic characterization (Saweljew et al. 1996). The BIOLOG® system also requires initial characterization such as Gram staining and morphology. Incorrect characterization of the bacteria will lead to false identification. For instance the Gram-positive isolate MAL-6 was identified as *S. kloosii* when read on a BIOLOG® GP plate but was incorrectly identified as *Yersinia pestis* when read on a BIOLOG® GN plate.

Conclusions

The fact that opportunistic microbial pathogens were found in these samples indicates the importance of monitoring ambient aerosols. Further, the antimicrobial-resistant nature of certain isolates could be of health concern. The different identification results found from the microbial methods applied here demonstrate the complexity of environmental sampling. In this work the objective was to examine viable cultures obtained from bioaerosols. While it is known that other factors including seasonality and weather conditions can impact aerosol testing results, more information is needed to determine the optimal microbial isolation and identification techniques for bioaerosols. In this study, we used culture techniques, FAME, BIOLOG®, biochemical with antibiotic senstivity testing, and 16S RNA sequencing to identify the bacteria from bioaerosols collected in three deiffernt locations and conclude that the results are valid.

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